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TITLE: The Role of U2AF1 Mutations in the Pathogenesis of Myelodysplastic Syndromes

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14. ABSTRACT
U2AF1 mutations occur in up to 11% of myelodysplastic syndrome (MDS) patients. To study the effects of the most common *U2AF1* mutation, U2AF1(S34F), on hematopoiesis and pre-mRNA splicing *in vivo*, we created doxycycline-inducible U2AF1(WT) and U2AF1(S34F) transgenic mice. Following transgene induction, U2AF1(S34F) mice have reduced WBCs, increased hematopoietic stem/progenitor cells, and increased HSC cell cycling compared to U2AF1(WT) mice. U2AF1(S34F) stem cells are at a competitive disadvantage compared to control cells, suggesting that the increase in HSC cell cycling following U2AF1(S34F) expression may lead to stem cell exhaustion. Next, we compared RNA splicing in progenitor cells from U2AF1(S34F) and U2AF1(WT) mice using whole transcriptome RNA-seq. We identified 460 splicing junctions that were differentially expressed in U2AF1(S34F) samples compared to U2AF1(WT). We validated several homologous dysregulated junctions (i.e., across species) in MDS patient bone marrow samples that have mutant U2AF1(S34F) versus U2AF1(WT). Together, these results suggest that mutant U2AF1 expression contributes to the altered hematopoiesis and pre-mRNA splicing observed in patients with *U2AF1* mutations. This study also identifies changes in gene isoform expression unique to *U2AF1* mutations that may have functional significance for MDS pathogenesis, which is being investigated in ongoing studies.

15. SUBJECT TERMS
Myelodysplastic Syndromes, Splicing, Spliceosome, Mouse Model, Hematopoiesis, RNA-seq, U2AF1

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1. INTRODUCTION:

The goal of this project is to understand the mechanism of disease pathogenesis induced by *U2AF1* mutations in myelodysplastic syndromes (MDS). U2AF1 is a key spliceosome protein that binds the AG dinucleotide of the 3' splice acceptor site in pre-mRNA introns during splicing and is mutated in up to 11% of MDS patients, making it one of the most commonly mutated genes in MDS. Overall, mutations in spliceosome genes occur in up to 57% of patients with MDS, further implicating altered pre-mRNA splicing in disease pathogenesis. We hypothesize that *U2AF1* mutations result in altered mRNA splicing in hematopoietic cells, and thereby lead to altered progenitor/stem cell function and ineffective hematopoiesis. In this project, we will test our hypothesis in the following Specific Aims. Specific Aim 1. We will determine whether the U2AF1(S34F) mutation alters hematopoiesis in vivo. We will inducibly express wild-type and S34F mutant (resulting from the most common *U2AF1* mutation) human *U2AF1* cDNAs in mice and determine the contribution of mutant U2AF1 to MDS pathogenesis by comprehensively evaluating basal hematopoiesis and stem cell function. Specific Aim 2. We will use RNA-Seq to identify alternatively spliced genes in primary hematopoietic progenitor cells harvested from U2AF1(S34F) mutant mice. We will identify alternatively spliced genes induced by *U2AF1* mutations by performing transcriptome sequencing (RNA-Seq) using RNA isolated from wild-type and mutant bone marrow progenitors. Candidate genes with alternative splicing will be interrogated in MDS patient samples with and without *U2AF1* mutations.

2. KEYWORDS:

Myelodysplastic Syndromes
Splicing
Spliceosome
Mouse model
Hematopoiesis
RNA-seq
U2AF1

3. OVERALL PROJECT SUMMARY:

Task 1. Seek IACUC and DoD ACURO approval for the use of animals.

Current Objectives: Obtain approval.
Results: IACUC and DoD ACURO approved.
Progress and Accomplishments with Discussion: Completed Task.

Task 2. Specific Aim 1. We will determine whether the U2AF1(S34F) mutation alters hematopoiesis in vivo.

Current Objectives: We will determine whether expression mutant U2AF1(S34F) induces ineffective hematopoiesis in mice. We will determine whether mutant U2AF1(S34F) contributes clonal dominance and MDS initiation.
Results: Following 4 weeks of transgene induction (and up to 12 months), U2AF1(S34F)-recipient mice have reduced total WBCs in the peripheral blood compared to U2AF1(WT)- and rtTA only-recipient controls (4.3 vs 7.11 and 7.13 K/μl, respectively, $p \leq 0.01$), but no significant changes in bone marrow cellularity or spleen size (n=9-11) (**Figure 1**).

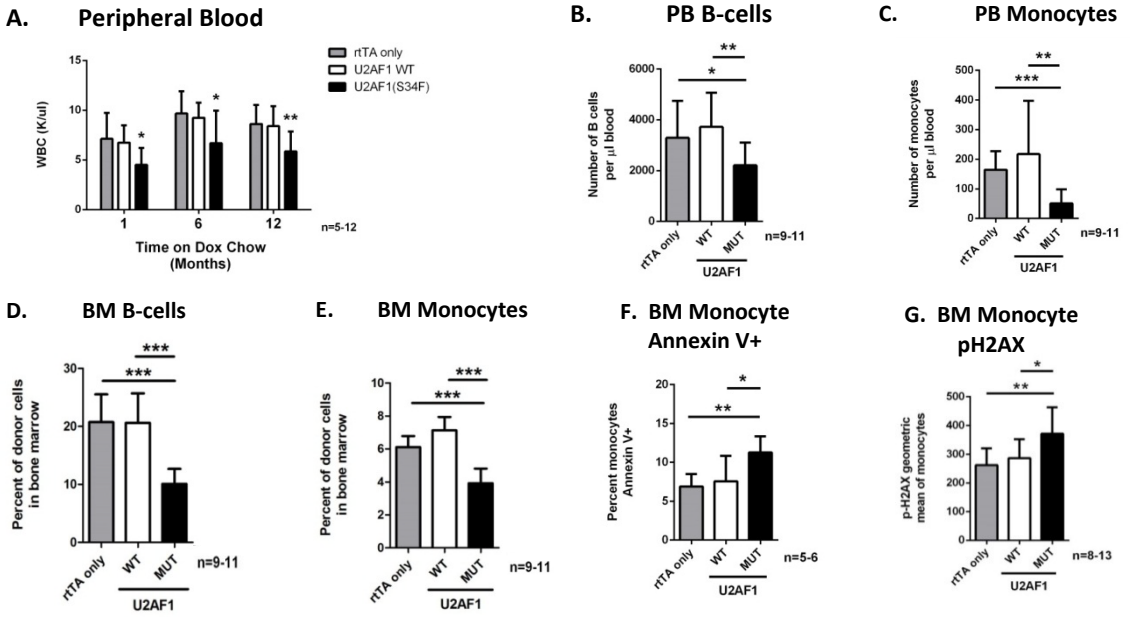

Figure 1. B-cells and monocytes are reduced in the peripheral blood and bone marrow of U2AF1(S34F) transgenic mice. A. WBC is reduced in U2AF1(S34F) mice. B-cells and monocytes are reduced at 1 month post-transgene induction in the peripheral blood (**B-C)** and bone marrow (**D-E).** Bone marrow monocytes have increased apoptosis at 1 month, measured by Annexin V (**F**) and phosphorylated H2AX (**G**). PB, peripheral blood. BM, bone marrow.). rtTA, reverse tetracycline-controlled transactivator . WT, wild-type. MUT, S34F mutant. * p<0.05, ** p≤0.01, ***p≤0.001

U2AF1(S34F)-recipient mice have a perturbed mature cell lineage distribution, including reduced monocytes and B cells in both peripheral blood ($p \leq 0.05$) and bone marrow ($p \leq 0.01$) when compared to control mice (n=9-11) (**Figure 1**). Reduction of bone marrow monocytes occurs as early as 5 days and is associated with increased Annexin V+ ($p \leq 0.05$) and phospho-H2AX ($p \leq 0.05$) compared to controls, suggesting loss of these cells may be due to apoptosis

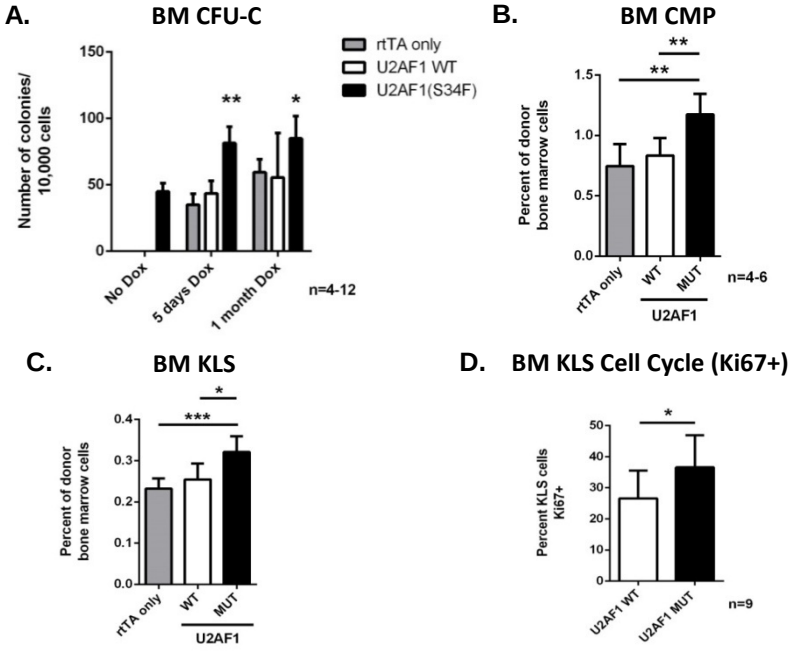

Figure 2. Bone marrow progenitors are increased in U2AF1(S34F) transgenic mice.
A. Increase in progenitors is seen 5 days following transgene induction in U2AF1(S34F) transgenic mice, as measured by methylcellulose assay. **B.** Common myeloid progenitors (CMP) are increased in U2AF1(S34F) mice, measured by flow cytometry at 1 month. **C.** Stem-cell enriched population (kit+/lineage-/Sca+; KSL) is increased in transgenic mutant mice, measured by flow cytometry at 1 month. **D.** KLS cells in the bone marrow are cycling more, as indicated by increased Ki67 staining. BM, bone marrow. rtTA, reverse tetracycline-controlled transactivator . WT, wild-type. MUT, S34F mutant. * p<0.05, ** p≤0.01, ***p≤0.001

(**Figure 1**). In addition, U2AF1(S34F)-recipient mice have increased numbers of progenitors in both bone marrow and spleen by CFU-C methylcellulose assay and flow cytometry for c-Kit+/Lineage- cells, as well as common myeloid progenitors (CMPs), when compared to U2AF1(WT) and rtTA only controls ($p \leq 0.05$, n=5-10) (**Figure 2**). U2AF1(S34F)-recipient mice also have an increase in the frequency of bone marrow hematopoietic stem cells (HSCs) measured by flow cytometry for bone marrow KLS (c-Kit+/Lineage-/Sca-1+) cells ($p \leq 0.05$) (**Figure 2**). The increase in bone marrow KLS cells in U2AF1(S34F)-recipient mice seen as early as 5 days is associated with higher levels of intracellular Ki67 (a marker of cell proliferation) in KLS cells compared to controls ($p<0.05$, n=8-13) (**Figure 2**).

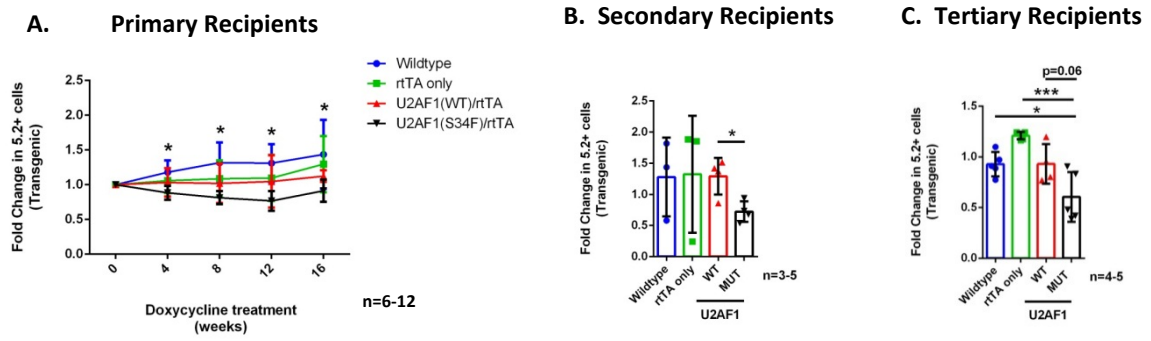

Figure 3. U2AF1(S34F) transgenic bone marrow is at a competitive disadvantage compared to normal bone marrow.
U2AF1(S34F) expressing bone marrow cells are at a comptetitive disadvantage compared to normal bone marrow in primary recipients (**A**), secondary recipients (**B**), and tertiary recipients (**C**). rtTA, reverse tetracycline-controlled transactivator . WT, wild-type. MUT, S34F mutant. * $p<0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Competitive repopulation studies show a disadvantage for bone marrow cells expressing mutant U2AF1(S34F) compared to U2AF1(WT) at ≥4 months post-transplant in both primary and secondary transplant recipient mice ($p \leq 0.05$, n=3-12) (**Figure 3**), suggesting that the increase in KLS cell cycling following U2AF1(S34F) expression may lead to stem cell exhaustion.
Progress and Accomplishments with Discussion: Collectively, these data indicate U2AF1(S34F) expression alters hematopoiesis *in vivo*.

Task 3. Specific Aim 2. We will use RNA-Seq to identify alternatively spliced genes in primary hematopoietic progenitor cells harvested from U2AF1(S34F) mutant mice.
Current Objectives: We will identify alternatively spliced genes in primary murine hematopoietic progenitor cells expressing mutant U2AF1 using RNA-Seq. We will validate alternatively spliced genes in primary MDS bone marrow cells expressing mutant U2AF1.
Results: We performed unbiased RNA sequencing on sorted bone marrow CMPs following 5 days of transgene induction in U2AF1(S34F)- and U2AF1(WT)-transplanted mice (n=3 each). We identified 460 splicing junctions that were differentially expressed in U2AF1(S34F) samples compared to U2AF1(WT) (FDR <5%). We observed a preference of the mutant U2AF1(S34F) to skip exons (p=1.3e-05, n=72) and alternative splice sites (p=0.014, n=45) with a T in the -3 position relative to the AG splice acceptor site of differentially-spliced genes (**Figure 4**); this effect has been previously reported in acute myeloid leukemia (AML) patient samples with U2AF1 mutations. To prioritize altered junctions for further analysis, we intersected mouse CMP junction results with RNA sequencing data from AML patient samples with and without U2AF1 mutations and primary human CD34+ cells expressing U2AF1(S34F) or U2AF1(WT). Across species and present in all 3 datasets, we identified homologous dysregulated junctions in 2 genes

known to be involved in cancer and stem cell biology: *H2AFY* and *MED24*. We validated concordant changes in both *H2AFY* and *MED24* isoform expression by RT-PCR using MDS patient bone marrow samples that have mutant U2AF1(S34F) versus U2AF1(WT) ($p<0.001$, n=5-6).

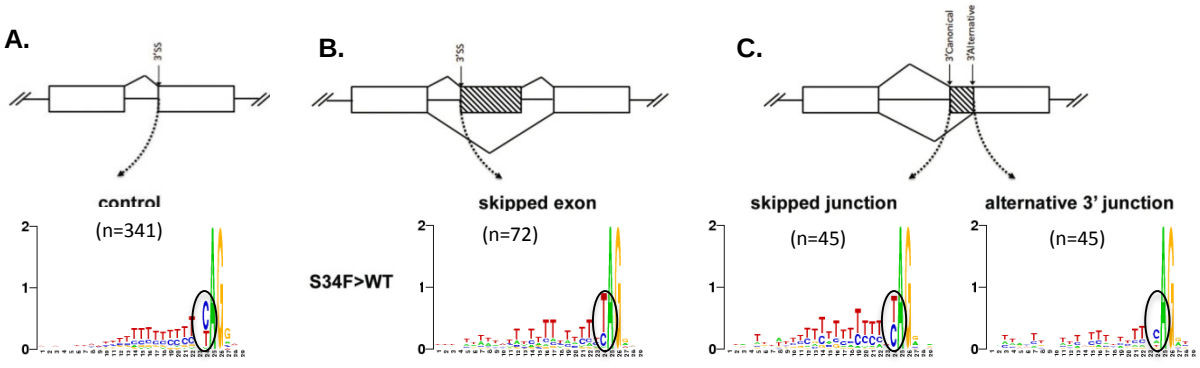

Figure 4. Mutant U2AF1(S34F) skips a "T" and splices into a "C" at the -3 position of splice-acceptor sites. **A.** Cartoon represents 2 exons separated by an intron in the top panel. The consensus sequence of 341 splice junctions that are spliced the same by U2AF1 mutant and wild-type expressing cells are shown in the logos plot (lower panel). A "C" preceding the AG splice acceptor site is common at the -3 position. **B.** Cartoon of a skipped exon (top panel) by mutant U2AF1(S34F) (middle hatched exon) tends to have a "T" > "C" at the -3 position (logos plot, lower panel) and differs from the control sequence in panel A. **C.** Cartoon of a cryptic 3' splice site within a downstream exon is shown in the top panel. The sequence skipped by mutant U2AF1 tends to have a "T" at the -3 position vs. a "C" for the alternative 3' junction that is spliced into (logos plot, lower panel). WT, wild-type.

Progress and Accomplishments with Discussion: Mutant U2AF1 expression contributes to the altered pre-mRNA splicing observed in patients with *U2AF1* mutations. This study also identifies changes in gene isoform expression unique to bone marrow samples expressing mutant U2AF1 that may have functional significance for MDS pathogenesis.

Task 4. Data analysis and report generation

Current Objectives: Analyze data.

Results: Analysis is ongoing, as reported above.

Progress and Accomplishments with Discussion: Anticipate completing analysis during year 2.

4. KEY RESEARCH ACCOMPLISHMENTS:

1. Determined that mutant U2AF1 expression alters hematopoiesis *in vivo*.
2. Determined that mutant U2AF1 expression alters pre-mRNA splicing in primary mouse hematopoietic cells.
3. Identified several genes that are alternatively spliced in both U2AF1 mutant expressing mice and human hematopoietic cells, prioritizing these genes as candidates that may contribute to disease pathogenesis.

5. CONCLUSIONS:

The results provide evidence that spliceosome gene mutations, specifically *U2AF1* mutations, affect hematopoiesis and may contribute to bone marrow failure. Given that spliceosome gene mutations are the most common family of genes mutated in MDS, a better understanding of the underlying mechanisms of disease pathogenesis will have broad implications. Future studies will focus on long-term monitoring of mice for development of bone marrow failure or leukemia. We are investigating the mechanism underlying how specific gene isoforms that are induced by

mutant U2AF1 alter hematopoiesis. We will also identify additional splicing alterations induced by mutant U2AF1 in various stem, progenitor, and precursor hematopoietic cell populations that will be validated in primary MDS samples. Our long-term goal is to identify a core set of genes that are perturbed during differentiation that contribute to bone marrow failure.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Published Manuscripts: Nothing to report.

Abstracts:

1. Shirai CL, Tibbitts J, Shao J, Ndonwi M, Ley JN, Kim S, Tripathi M, Okeyo-Owuor T, Graubert TA, Walter MJ. Mutant U2AF1 Expression Alters Hematopoiesis In Transgenic Mice. American Society of Hematology, 55th Annual meeting, New Orleans, LA, USA. December 9, 2013.

Presentations:

1. Memorial Sloan-Kettering Cancer Center, Hematology/Oncology Grand Rounds, New York, NY, USA. Title: Spliceosome Gene Mutations in MDS. January 14, 2014.
2. Aplastic Anemia & MDS International Foundation (AA&MDSIF), 2014 Bone Marrow Failure Disease Scientific Symposium, Bethesda, MD, USA. Title: Pathophysiology and New Molecular Targets in MDS. March 27, 2014.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: Nothing to report.

9. OTHER ACHIEVEMENTS: Nothing to report.

10. REFERENCES: None.

11. APPENDICES: None.